

15413/83 COMMONWEALTH of AUSTRALIA

PATENTS ACT 1952

LODGE
10 JUN 1982
AUSTRALIAN
PATENT OFFICE

SECTION 34(4)(a) DIRECTION SEE FORM STANDARD PATENT

NAME DIRECTED *HOWARD FLOREY INSTITUTE OF EXPERIMENTAL
PHYSIOLOGY AND MEDICINE, UNIVERSITY OF MELBOURNE, PARKVILLE,
VICTORIA, COMMONWEALTH OF AUSTRALIA.*

XX GEOFFREY WILLIAM TREGEAR, of 62 Hawthorn Grove, Hawthorn, Victoria,
We in the State of Victoria, Australia, DU YU-CANG, of
Shanghai Institute of Biochemistry, Academia Sinica, Shanghai,
The People's Republic of China and HUGH DAVID NIAL, of 3 Bendigo
Avenue, Elwood, in the State of Victoria, Australia

COMPLETE AFTER PROVISIONAL SPECIFICATION No. 15413/83

hereby apply for the grant of a Standard Patent for an invention entitled:

"PEPTIDES WITH RELAXIN ACTIVITY"

which is described in the accompanying provisional specification.

Details of basic application(s):

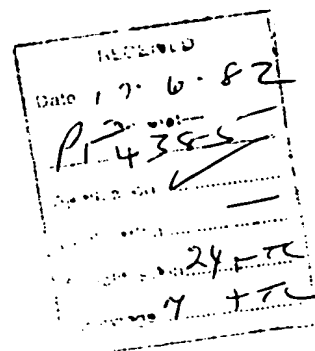
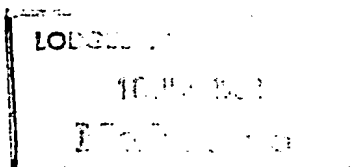
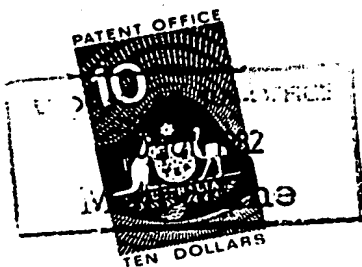
Number

Convention Country

Date

APPLICATION ACCEPTED AND AMENDMENTS

ALLOWED 23.3.87



The address for service is care of DAVIES & COLLISON, Patent Attorneys, of 1 Little
Collins Street, Melbourne, in the State of Victoria, Commonwealth of Australia.

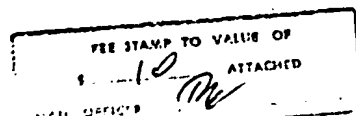
Dated this

10th

day of

June

1982



TO: THE COMMISSIONER OF PATENTS

H. W. Rimington
(a member of the firm of DAVIES &
COLLISON for and on behalf of the Applicant).

Davies & Collison, Melbourne and Canberra.

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COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952
DECLARATION IN SUPPORT OF CONVENTION OR
NON-CONVENTION APPLICATION FOR A PATENT

In support of the Application made for a patent for an invention
entitled: **"PEPTIDES WITH RELAXIN ACTIVITY"**

Insert title of invention.

Insert full name(s) and address(es)
of Declarant(s) being the appli-
cant(s) or person(s) authorized to
sign on behalf of an applicant
company.

I **ROBERT HOOKHAM**, Business Manager & Secretary,
of **Howard Florey Institute of Experimental
Physiology and Medicine**, a body corporate
established under the laws of the State of
Victoria, of the University of Melbourne,
Parkville, in the State of Victoria,
Commonwealth of Australia,

do solemnly and sincerely declare as follows:-

1. ~~I am~~ ^{I am} the applicant..... for the patent

or (b) I am authorized by **HOWARD FLOREY INSTITUTE OF EXPERI-
MENTAL PHYSIOLOGY AND MEDICINE**

Cross out whichever of paragraphs
1(a) or 1(b) does not apply.

1(a) relates to application made
by individual(s).

1(b) relates to application made
by company; insert name of
company.

Cross out whichever of paragraphs
2(a) or 2(b) does not apply.

2(a) relates to application made
by inventor(s)

2(b) relates to application made
by company(s) or person(s) who
are not inventor(s); insert full
name(s) and address(es) of inven-
tors.

the applicant..... for the patent to make this declaration on ~~their~~ ^{its} behalf.

2. ~~I am~~ ^{I am} the actual inventor..... of the invention

or (b) **GEOFFREY WILLIAM TREGEAR**, of 62 Hawthorn Grove,
Hawthorn, Victoria 3122, Commonwealth of
Australia;

DU YU-CANG, of Shanghai Institute of Biochemistry,
Academica Sinica, Shanghai, The People's
Republic of China; and

HUGH DAVID NIALI, of 3 Bendigo Avenue, Elwood,
Victoria 3184, Commonwealth of Australia

~~is~~ ^{are} the actual inventor..... of the invention and the facts upon which the applicant.....

~~is~~ ^{are} entitled to make the application are as follows:- the said actual
inventors assigned their right in the invention to
Relaxin Pty. Ltd., who in turn assigned the
rights to **Howard Florey Institute of Experimental
Physiology and Medicine**.

State manner in which appli-
cant(s) derive title from inven-
tor(s).

Cross out paragraphs 3 and 4
for non-convention applications.
For convention applications
insert basic country(s) followed
by date(s) and basic applicant(s).

3. The basic application..... as defined by Section 141 of the Act ^{was} ~~were~~ made

in on the
by
in on the
by
in on the
by

4. The basic application..... referred to in paragraph 3 of this Declaration ^{was} ~~were~~
the first application..... made in a Convention country in respect of the invention the subject
of the application.

Insert place and date of signature.

Declared at **MELBOURNE** this **THIRTEENTH** day of **JUNE, 1966**

Signature of Declarant(s) (no
attestation required).

Note: Initial all alterations.

DAVIES & COLLISON, MELBOURNE and CANBERRA.

(12) AUSTRALIAN PATENT ABRIDGMENT

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(54) PEPTIDES WITH RELAXIN ACTIVITY

(71) HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND
MEDICINE

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(57) Claim

1. A method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains, or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic, water-miscible solvents, washing the mixed peptides; oxidizing the mixed peptides in the presence of a non-reactive, water soluble, inorganic salt; and isolating the relaxin thus produced.

9. A synthetic porcine relaxin analogue consisting of the A(1-22) and B(1-25) peptide chains.

22. A porcine relaxin analogue which has been modified by one or more of the following procedures:

(a) the addition of one or more amino-acids to one or

both of the natural A and B chains;

(b) the deletion of one or more amino acids from the natural B and/or A peptide chains;

.../2

(c) the chemical modification of one or more amino acids

(d) the replacement of at least one of the natural amino-acids in one or both of the A and B chains with a different amino acid

of the natural B and/or A peptide chains;
with the proviso that the thus modified porcine relaxin analogues possess porcine relaxin activity.

561670

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(Original)

FOR OFFICE USE

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15413/83

Complete Specification Lodged:
Accepted:
Published:

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Related Art:

and is correct in its details.

HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY
AND MEDICINE

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LODGED AT SUB-OFFICE
- 6 JUN 1983
Melbourne

Complete specification for the invention entitled:

"PEPTIDES WITH RELAXIN ACTIVITY"

The following statement is a full description of this invention,
including the best method of performing it known to us :-

This invention relates to the synthesis of peptides with relaxin activity. The invention is particularly concerned with the synthesis of porcine relaxin and some structural analogues.

5 The ovarian peptide hormone relaxin plays an important role in pregnancy and parturition. The main biological action of the hormone is to soften and lengthen the inter-pubic ligaments: it also dilates the cervix and inhibits contractions of the uterus.

10 Relevant prior art is contained in the references cited herein which are identified by numbers in brackets in the text and are collected together at the end of this description. Other prior art is collected and described in the specification of British Patent
15 No. 2,072,680 (equivalent to U.S. Serial No. 134,390, filed March 27, 1980). The content of these references is hereby incorporated into this specification.

20 The complete amino acid sequence of relaxin extracted from the ovaries of the pregnant pig has been recently established (1, 2) and is shown in Figure 1 of the accompanying drawings. The amino acid sequence of rat relaxin has also been elucidated (3, 4) and Gowan et al. (5) have reported a partial sequence analysis of the hormone from the shark ovary.

The general structural features of the relaxin molecule bear striking resemblance to insulin. Relaxin is composed of two peptide chains (A and B) joined by disulfide bonds through the cysteines at A9-B10 and A22-B22 (see Figure 1), with an intra-chain disulfide bridge within the A-chain between A8 and A13. The disposition of the disulfide bonds is thus analogous to that of insulin. A model of the relaxin sequence can be accommodated without strain into the three-dimensional coordinates of the insulin structure (6, 7) and circular dichroism analyses of the hormones in solution are very similar (8, 9). Thus relaxin and insulin may share common features of secondary and tertiary structure even though the hormones have no measurable overlap in biological or immunological properties (9).

Three forms of relaxin of equivalent biological activity, which are characterized by their elution behaviour on carboxymethyl cellulose chromatography, have been isolated from the ovaries of the pregnant pig. These forms of relaxin have been designated CMB, CMa' and CMa by Sherwood & O'Bryne (10). Sequence analysis has revealed that these three forms of pig relaxin have an identical A chain of 22 amino acids but differ in sequence at the carboxyl terminus of the B chain (12). CMa relaxin has a B-chain of 31 amino acids; the B-chains of CMa' and CMB are shorter, having 29 and 28 residues respectively (see Figure 1).

The predominant form of the hormone stored in the pig ovary is the CMa or B31 variant: the CMB and CMa' peptides are probably largely generated during the isolation procedures (11).

We have carried out research into the chemical synthesis of relaxin and its structural analogues as part of a systematic study of the structure-function relationships in the hormone molecule. The purified
5 separate chains of pig relaxin have been obtained in approximately 10% yield (based on crude). The major impurities in the synthetic peptides appear to have arisen through side-reactions of the cysteine residues occurring during the HF cleavage step. In more recent
10 syntheses improved yields have been obtained using the HF stable S-ethyl mercapto protecting group for cysteine. We have found that both the natural and synthetic B chains of pig relaxin, whether in the reduced or S-sulfonated form, are particularly
15 difficult to dissolve in aqueous solution. In addition, the relaxin B chains exhibit unusual adsorptive properties. Column chromatography on Sephadex, cellulose, polyamide or polystyrene resins results in virtually a complete loss of peptide by
20 irreversible absorption (12). These properties have made purification of synthetic relaxin B chain peptides and their coupling to A chain particularly difficult. Detailed studies of the conformation of the relaxin B chains by circular dichroism have revealed that the
25 configuration of the B31, B29 and B28 peptides in solution were largely beta-structure.

We have found that conditions which give efficient combination of the insulin A and B chains (13) give
30 very low or negligible yields when use for the structurally related relaxin. Our investigations have now shown that precipitation of the mixed relaxin peptide chains with a neutral water miscible solvent such as acetone, washing to remove reducing agent, and

the addition of a water soluble inorganic salt such as NaCl during the oxidation (recombination) step are essential requirements.

5 It is an object of the present invention to provide methods for the preparation of relaxin, particularly porcine relaxin and its structural analogues which provide improved product yields over the known art.

10 More specifically, one object of the invention is to provide a method for joining the A and B chain peptides of natural and/or synthetic porcine relaxin.

Another object is to provide a method of producing relaxin analogues by combining modified forms of the natural B and/or A relaxin chains.

15 A further object is to provide modifications and/or structural analogues of natural porcine relaxin.

Other aims and objects of the invention will be apparent from the following description.

20 In accordance with one aspect of the present invention, there is provided a method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic,
25 water-miscible solvent; washing the mixed peptides; oxidizing the mixed peptides in the presence of a

non-reactive water soluble inorganic salt; and
isolating the relaxin thus produced.

The above described method is particularly
applicable to the synthesis of porcine relaxin, however
5 it is also applicable to other types of relaxin,
including human relaxin.

The term "non-reactive" as used throughout this
description and claims is used to indicate that the
atmosphere, solvent, or salt, or the like, is not
10 chemically reactive with peptides.

The neutral, water-miscible, organic solvent used
to precipitate the reduced peptides may be any such
solvent known per se as a precipitant for peptides.
Suitable solvents include the lower aliphatic ketones
15 and alcohols, with acetone being the preferred solvent.

Any non-reactive, water-soluble inorganic salt may
be present during the oxidation or recombination step.
The use of sodium chloride is, however, preferred.

Preferably, the isolation and purification of the
20 product is carried out using chromatographic methods.

More specifically in accordance with this aspect
of the invention, a method for the synthesis of relaxin
comprises the steps of:

separately preparing or isolating the S-sulfonated
25 A and B peptide chains;

forming a mixture of the S-sulfonated A and B peptides;

5 reducing the mixture at pH 7 to 9, preferably 8.3, under a non-reactive atmosphere, preferably nitrogen, for at least 6 minutes;

adjusting the pH of the reduced mixture to 4.5 to 5.5, preferably pH5, preferably with acetic acid;

10 adding a non-reactive, neutral, water-miscible, organic solvent, preferably acetone, to the mixture to precipitate the mixed peptides;

washing the mixed peptides with a suitable solvent, preferably ethyl acetate and ether, to remove the reducing agent;

15 oxidizing the mixed peptides at pH 9.5 to 11, optimally at about 10.4 to 10.6, for about 48 to 72 hours at about 0° to 10°C, preferably 5°C, in the presence of a non-reactive, water soluble, inorganic salt, preferably sodium chloride, in a concentration of at least about 0.1M, preferably about 0.5 to 1M.

20 Throughout this specification reference will be made to the accompanying drawings in which:-

Figure 1 shows the amino-acid sequences of the A and B chains of porcine relaxin;

25 Figure 2 shows diagrammatically, a purification procedure for a crude synthetic S-sulfonated porcine relaxin B-chain;

Figure 3 shows circular dichroism (CD) spectra for:-

- (a) native porcine relaxin;
- (b) the S-sulfonated, and reduced (un-sulfonated) A chains;
- (c) S-sulfonated, full-length and shortened B chains;
- (d) combinations and mixtures of various A and B chains;

Figure 4 shows the results of typical purification procedures as described herein; and

Figure 5 shows the results for the final ion-exchange purification stage of a synthetic (A22 B25) combination mixture.

Using the above-described method, the natural A(1-22) and B(1-28) chains were combined in yields of up to 25% (based on the B chain). The combination yield for synthetic A(1-22) and B(1-28) chains was 0.7%.

We have also found that the combination yields of the synthetic A and B chains can be improved by shortening of the B chain at the carboxyl terminus to as little as 25 residues. As shown by Figure 3(c), the CD spectra indicate that shortening to 25 residues results in a change of structure from the beta-configuration to an unordered conformation. This conformational change is reflected in an enhanced solubility of the peptide in aqueous solution, a loss of the adsorptive behaviour and an improvement in the combination yield with the A chain. Using the

above-described method the combination yield rises to 10%. The specific activity of the A22 B25 peptide as so prepared is 33% of that of the natural A22 B31 peptide. After purification, the A22 B25 peptide has a specific activity of 93% of the native A22 B31 relaxin. As shown in Figure 3(d) the CD spectrum of the A22 B25 peptide closely resembles that of native relaxin (Figure 3(a)).

Shortening is also tolerated at the amino terminus of both the A and B chains as well as at the carboxyl terminus of the B chain. The B(4-23)NH₂ chain has a similar CD spectrum (Figure 3(c) to the B(1-25) chain. The synthetic peptide A(4-22)B(4-23)NH₂ still retains significant biological activity.

Thus in accordance with another aspect of this invention, there is provided a relaxin analogue, particularly a porcine relaxin analogue, consisting essentially of shortened forms of the natural B and/or A peptide chains.

This aspect of the invention also provides a method for producing a relaxin analogue, particularly a porcine relaxin analogue, which comprises the step of forming the shortened B and/or A peptide chains and combining them by the method of the invention as described above.

The preferred shortened porcine relaxin peptide chain combinations are A(1-22) and B(1-25).

A further aspect of the present invention provides for chemical modification of the B and/or A chains (in

either full-length or shortened form) prior to combination by the method of the invention. Two types of chemical modification may be employed, either singly or in combination.

- 5 The first type involves the modification of one or more of the amino-acids which occur in the natural B and/or A chains. Such modification will generally involve protection of active groups on one or more of the amino-acids by methods known per se, and the
10 protecting groups may, if desired, be removed after combination of the (modified) A and B chains.

- Examples of this type of modification include the acetylation or similar protection of free amino groups, amidation of C-terminal groups, or the formation of
15 esters of hydroxyl or carboxylic groups. The formyl group is a typical example of a readily-removable protecting group.

- The second type of modification includes replacement of one or more of the natural amino-acids
20 in the B and/or A chains with a different amino acid (including the D-form of a natural amino-acid). This general type of modification may also involve the deletion of a natural amino-acid from the chain or the addition of one or more extra amino-acids to the chain.

- 25 The purpose of such modifications is to enhance the combination yields of the A and B chains, while maintaining the activity of the product, i.e., relaxin or an analogue thereof, or to enhance the activity of the product for a given combination yield.

A specific example of the first type of modification is acetylation of the N-terminal amino group of the B chain (B28, B25 or B23) or the modification of a tryptophan residue by addition of a formyl group.

Examples of the second type of modification are (i) replacement of the Trp moiety at B27 with glycine (Gly), (ii) replacement of the Pca moiety at B1 with glutamine (Gln) or glutamic (Glu) and (iii) replacement of Met moiety at A2 with norleucine (Nle).

An example showing both types of modification is replacement of the Pca moiety at B1 with N-acetylglutamine.

The invention in this aspect also includes relaxin analogues formed from natural or shortened B and/or A chains modified in accordance with the invention as described above.

The invention, in its various aspects, is further described and elucidated in the following examples.

20 EXAMPLE 1

(a) Starting Materials

The three structural variants of native relaxin (A22B28, A22B29 and A22B31) were isolated from pregnant pig ovaries and purified according to the procedure described by Sherwood and O'Byrne (10). Purified A22B31 peptide was also prepared by the method of Walsh and Niall (11).

(b) Separation of insulin and relaxin chains by S.sulfonation.

The A and B chains of porcine insulin were separated by S-sulfonation and purified according to the procedure previously described (13). For the S-sulfonation and separation of relaxin chains, the following modification to this procedure was used. Native porcine relaxin (A22B29, 19.8mg) was dissolved in 4ml tris buffer (0.05M, pH 8.3). To this solution was added sodium sulfite (28mg) and sodium tetrathionate (14mg) and the mixture stirred at 37°C. After 3.5 hours further sodium sulfite (14mg) and sodium tetrathionate (7mg) were added and the mixture stirred at 37°C for an additional 3 hours and then left to stand at room temperature overnight. The precipitated B-chain S-sulfonate was separated from A-chain S-sulfonate by centrifugation at 3000 rpm for 10 minutes. The B-chain sulfonate was partially purified by dissolving in dilute ammonia solution (5 ml) and precipitating at pH5 by the addition of glacial acetic acid, followed by centrifugation. This procedure was repeated twice. The precipitate was then redissolved in dilute ammonia solution and lyophilized to yield 11mg of B29 S-sulfonate. The supernatant solutions obtained from the above procedure were combined and dialyzed for 2 days against distilled water and then lyophilized to yield 6.3 mg of S-sulfonated A-chain.

The above procedure was also used to separate the A and B chains of porcine relaxin A22B28 and A22B31.

The time course of S-sulfonation for relaxin and insulin peptides was followed by titration with

p-chloromercuriobenzoic acid using procedures previously described (17).

When required, the S-sulfonated relaxin A-chain was further purified by ion exchange chromatography on DEAE-Sephadex A25 in tris-HCl buffer pH 8.5 using a sodium chloride gradient from 0 to 0.5M. Attempts to purify S-sulfonated relaxin B-chain by ion-exchange chromatography generally resulted in a complete loss of peptide by adsorption to the column. A reasonably satisfactory purification of B-chain was achieved by dissolving the S-sulfonated mixture in 8M urea formic acid pH 3.0 and applying the sample to a Dowex 5DWX2 ion-exchange column and eluting with a stepwise sodium chloride/urea gradient. Under normal conditions one would expect the S-sulfonated B-chain to elute first from the column. In practice, the B-chain adsorbed strongly to the Dowex and allowed the S-sulfonated A-chain to be eluted first. Purified S-sulfonated B-chain could be recovered, although in poor yield, by stripping the column with dilute ammonia solution at pH 9.5. Following dialysis and a precipitation-washing cycle at pH 9 and pH 3.8, S-sulfonated B-chain was obtained free from A-chain and sodium sulfite.

The S-sulfonated chains of relaxin were characterized by electrophoresis on cellulose acetate paper in phosphate buffer pH 7.8 (2000 V. for 20 minutes). The peptide spots were visualized by oxidation.

EXAMPLE 2Synthesis of Porcine Relaxin A Chain

The 22-peptide chain representing the sequence of
 porcine relaxin A chain was assembled on a
 5 phenylacetamidomethyl - 1% cross linked polystyrene
 resin support (16) using standard solid-phase
 procedures (12). The amino acid side-chain functional
 groups were protected as follows: serine and threonine
 as the O-benzyl ether, aspartic and glutamic acids as
 10 the benzyl ester, arginine as the p-toluenesulfonyl-,
 lysine as the 2-chlorocarbobenzoxy- and cysteine as the
 p-methoxybenzyl-derivatives respectively. The
 completed peptide was cleaved from the resin support
 with hydrogen fluoride in the presence of anisole (10%)
 15 for 30 minutes at 0°C. Exposure of the peptide to
 hydrogen fluoride was minimized to prevent formation of
 the glutamic acid anisole adduct. The crude peptide
 was reduced with dithiothreitol in 6M guanidine
 hydrochloride and applied to a BioGel P10 column in 30%
 20 acetic acid. Further purification was by ion-exchange
 chromatography on SP-Sephadex with a pyridine-acetic
 acid gradient pH 2.5 to pH 5.0. The major peak from
 the SP-Sephadex column was S-Sulfonated (15) and
 applied to DEAE-Sephadex. The S-Sulfonated peptides
 25 were eluted with a linear gradient of NaCl to 0.5 M in
 tris-buffer (0.05M), pH 8.3 (Figure 4(a)). Relaxin A
 (1-22) SSO_3^- eluted at a conductivity of 11 to 13 mS
 and was characterized following dialysis by amino acid
 analysis, HPLC (see Figure 4(d)) and paper
 30 electrophoresis. The overall yield of purified Relaxin
 A (1-22) SSO_3^- was generally of the order of 10% based
 on the initial amount of crude peptide.

EXAMPLE 3Synthesis of Relaxin A Chain Analogue

5 In a separate synthesis, using the general procedures described in Example 2, an analogue of the relaxin A-chain was prepared wherein the methionine at position 2 was replaced with the isosteric norleucine residue.

EXAMPLE 4Synthesis of Porcine Relaxin B-Chain

10 Initially, the synthesis of CMa' or B(1-29) was attempted using the same general strategy outlined for the A-chain synthesis in Example 2. The crude B(1-29) peptide exhibited unusual solubility characteristics. Although the crude peptide dissolved completely in
15 dithiothreitol-guanidine-30% acetic acid, the vast majority of the peptide was lost by adsorption or precipitation following gel filtration on BioGel or Sephadex. The B-chain peptide was soluble above pH 5.5 but precipitated out of solution on ion-exchange
20 columns when eluted with a salt gradient. The salting-out or adsorptive effect of the synthetic B-chain occurred whether the peptide was in the reduced or S-Sulfonated form and severely hampered attempts to purify the peptide by conventional chromatography.
25 Adequate purification of the synthetic B-chain could only be effected by precipitating the peptide at pH 5 and washing away the soluble impurities. A marked improvement in the solubility of the relaxin B-chain was noted when the B(1-29) peptide (CMa') was converted
30 to B(1-28) (CMB) by removal of the carboxyl terminal arginine residue with Cpase B. The purification scheme

used for the synthetic S-Sulfonated relaxin B-chain, which resulted in a 15 - 20% yield based on the initial crude peptide, is outlined in Figure 2.

- 5 A convenient method for monitoring the purification of the synthetic relaxin A and B chains was found to be paper electrophoresis on cellulose acetate in phosphate buffer at pH 7.8.

EXAMPLE 5

(a) Synthesis of Relaxin B Chain Analogues

- 10 The B chain analogues set out in Table 1 below were prepared using the general procedures described in Example 2. Figures 4(b) and 4(c) show the results obtained in the first and final purification stages respectively for the synthetic S-sulfonated B(1-25)
15 chain.

(b) Synthesis of [Gly²⁷] relaxin B-chain Analogue

- 20 The glycine-27 analogue of porcine relaxin B-chain in which the tryptophan which normally occurs at position 27 was replaced by glycine, was synthesized by the general procedures described in Example 2. The other tryptophan at position 18 in the porcine B-chain sequence was retained.

- 25 A modification of the procedures described in Example 2 was the addition of 0.2% indole to the acidic α -amino deprotection reagent following incorporation of the tryptophan-18 residue into the synthetic chain.

Following cleavage of the assembled peptide from the solid support the crude peptide was purified by gel filtration, ion exchange chromatography and preparative HPLC. In contrast to the porcine relaxin B(1-28) peptide, the reduced and S-sulfonated [Gly²⁷] analogue peptide did not adsorb to the chromatography media and could be effectively purified by these methods.

Circular dichroism analysis of the [Gly²⁷] analogue peptide gave spectra characteristic of a random coil configuration. This is in contrast to the native and synthetic relaxin B(1-28) peptide which were at least 90% of beta structure. These results suggest that the conformational and solubility characteristics of relaxin B-chain peptides are related to hydrophobic interactions involving the tryptophan residue at position 27.

The synthetic [Gly²⁷] porcine relaxin B(1-28) peptide was combined with synthetic porcine relaxin A-chain according to the procedures described in Example 6 (below). The chain combination mixture was biologically active in the in vitro rat uterine contractility assay and the in vivo guinea pig inter pubic ligament assay.

EXAMPLE 6

25 (a) The recombination reaction

Initially the S-sulfonated relaxin A and B chains were recombined using conditions previously found to be optimal for the recombination of insulin chains (18). As the recombination yields obtained for relaxin were

low using this general procedure (of the order of 1 to 2%), we investigated the solubility characteristics of the relaxin A and B chains, the influence of chain length of the relaxin B chain, the effect of the addition of urea, sodium chloride or dioxane to the recombination mixture, the ratio of A and B chains and the time of oxidation. The conformation of the relaxin A and B chains in solution and the structural changes occurring during chain combination were followed by circular dichroism spectroscopy (19).

(b) Preferred conditions for the recombination of porcine Relaxin A and B chains

S-sulfonated relaxin A chain (1.0 mg) and S-sulfonated relaxin B chain (1.0 mg) were dissolved in 0.05M tris-HCl buffer pH 8.3 (0.2 ml) in a stoppered test tube and the solution cooled to 0°C in an ice bath. A solution of mercaptoethanol in water (1.4N) was adjusted to pH 8 by the dropwise addition of dilute ammonia solution and 0.12ml added to the solution of relaxin chains. The mixture was agitated and degassed three times with oxygen free nitrogen and placed in a 37°C water bath for 5.5 minutes. The solution was then adjusted to pH 4.5 with 30% acetic acid and the peptide chains precipitated by the addition of acetone (4 ml). The mixture was cooled to 0°C for 5 minutes then centrifugated. The precipitate was then washed further with acetone (4 ml), ethyl acetate (4 ml) and finally petroleum ether (4 ml) and dried in vacuo. The precipitate was then dissolved in degassed water (0.2 ml) previously brought to pH 10 with the addition of 0.5N sodium hydroxide. To this solution was added 0.05M glycine buffer pH 10.6 (0.4ml) and sodium

chloride to give a final concentration of 1M and the mixture agitated and allowed to stand for 3 days at 4°C. The reaction mixture was centrifuged if necessary, and the peptide content in the supernatant measured by UV absorption at 280 nm prior to the assessment of the biological activity.

(c) Characterization of recombined peptide chains.

The recombination of the relaxin peptide chains was monitored by HPLC and radioimmunoassay using an antibody raised to native porcine relaxin A22B29 and I125 Bolton-Hunter labelled A22B29 tracer (22). The biological activity of the recombined products was assessed using the rat uterine contractility assay (14).

The biological activity of the synthetic relaxin peptides was tested in the rat uterine contractility assay (14).

Table 1 shows the actual combination yields (measured by bioassay) obtained for various combinations of the natural (N) and synthetic (S) A and B chains and various synthetic analogues. Column 3 shows the actual combination yields (based on the B chain). Column 4 shows the yields recalculated on the basis of 100% yield for the recombined natural A22 and B28 chains.

The following points are noted.

- (i) The recombination yield for the synthetic A22 B28 chain is very much lower than that for the natural

chain. This is attributed to a lack of homogeneity in the synthetic peptides.

- 5 (ii) Recombination yields for the synthetic peptides are increased by shortening the B chain to 25 residues and/or by replacement of certain residues in the B and/or A chains.

(iii) Biological activity is retained even when the A and B chains are shortened at both termini.

TABLE 1

<u>A-Chain</u>	<u>B-Chain</u>	<u>Recombination Yield</u> (by BIOASSAY)	
NA (1-22)	NB (1-28)	25%	100%
SA (1-22)	SB (1-28)	0.7	3
SA (1-22)	SB[GLU ¹] (1-28)	2.1	8
SA (1-22)	SB[acetyl GLU ¹] (1-28)	2.1	8
SA (1-22)	SB (1-25)	5.0-7.0	24
SNle ² A (1-22)	SB (1-28)	1.5	6
SNle ² A (1-22)	SB[GLU ¹] (1-28)	2.5	10
SNle ² A (1-22)	SB[acetyl GLU ¹] (1-28)	1.6	6
SNle ² A (1-22)	SB (1-25)	4.0	16
SA (1-22)	SB (1-28)NH ₂	1.7	7
SA (1-22)	SB (1-23)NH ₂	0.8-2.0	6
SA (1-22)	SB[N-acetyl] (4-23)NH ₂	2.4	10
SA (4-22)	SB[acetyl GLU ¹] (1-28)	1.0	4
SA (4-22)	SB (1-25)	2.4	10
SA (4-22)	SB (1-23)NH ₂	1.3	5
SA (4-22)	SB[N-acetyl] (4-23)NH ₂	1.3	5
SA (1-22)	SB[Gly ²⁷] (1-28)	3.0	12
SA (1-22)NH ₂	SB[Gly ²⁷] (1-28)	2.4	10
SA (7-22)	SB[N-acetyl] (4-23)NH ₂	0	0

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for the synthesis of relaxin or modified forms or analogues thereof, which comprises the steps of reducing a mixed solution of the S-sulfonated A and B peptide chains, or modified forms or analogues thereof; precipitating the reduced peptides with a non-reactive, neutral, organic, water-miscible solvents, washing the mixed peptides; oxidizing the mixed peptides in the presence of a non-reactive, water soluble, inorganic salt; and isolating the relaxin thus produced.

2. A method as claimed in Claim 1, wherein said peptide chains are porcine relaxin peptide chains.

3. A method as claimed in Claim 1 or Claim 2, which comprises the steps of:

separately preparing or isolating the S-sulfonated A and B peptide chains;

forming a mixture of the S-sulfonated A and B peptides;

reducing the mixture at pH of ~~about~~ 7 to ~~about~~ 9 under a non-reactive atmosphere for at least 6 minutes;

adjusting the pH of the reduced mixture to 4.5 to 5.5;

adding said non-reactive, neutral, water miscible solvent to the mixture to precipitate the mixed peptides;

washing the mixed peptides with a suitable solvent to remove the reducing agent;

oxidizing the mixed peptides at a pH of ~~about~~ 9.5 to ~~about~~ 11 for ~~about~~ 48 to 72 hours at a temperature of ~~about~~ 0° to 10°C in the presence of said

non-reactive water soluble inorganic salt in a concentration of at least 0.1M.

4. A method as claimed in Claim 3, wherein said non-reactive atmosphere is nitrogen.

5. A method as claimed in any one of Claims 1 to 4, wherein said neutral, organic, water-miscible solvent is acetone, and said non-reactive water soluble inorganic salt is sodium chloride.

6. A method as claimed in any one of Claims 1 to 5, wherein a shortened form of one or both of the A and B chains is used.

7. A method as claimed in any one of Claims 1 to 5, wherein at least one amino-acid in one or both of the A and B chains is chemically modified prior to combination.

8. A method as claimed in Claim 7, wherein the chemical modification comprises the addition of a protective group to a free amino group, and the protective group is optionally removed following combination.



9. A synthetic porcine relaxin analogue consisting of the A(1-22) and B(1-25) peptide chains.

10. A synthetic porcine relaxin analogue consisting of A (1-22) and B [GLU¹](1-28) peptide chains.

11. A synthetic porcine relaxin analogue consisting of the A (1-22) and B [acetyl GLU¹](1-28) peptide chains.

12. A synthetic porcine relaxin analogue consisting of the A[Nle²](1-22) and B[GLU¹](1-28) peptide chains.

13. A synthetic porcine relaxin analogue consisting of the A[Nle²](1-22) and B[acetyl GLU¹](1-28) peptide chains.

14. A synthetic porcine relaxin analogue consisting of the A[Nle²](1-22) and B (1-25) peptide chains.

15. A synthetic porcine relaxin analogue consisting of the A (1-22) and B (1-28)NH₂ peptide chains.

16. A synthetic porcine relaxin analogue consisting of the A (1-22) and B (1-23)NH₂ peptide chains.

17. A synthetic porcine relaxin analogue consisting of the A (1-22) and B[N-acetyl]{4-23}NH₂

peptide chains.

18. A synthetic porcine relaxin analogue consisting of the A (4-22) and B[acetyl GLU¹](1-28) peptide chains.

19. A synthetic porcine relaxin analogue consisting of the A (4-22) and B (1-25) peptide chains.

20. A synthetic porcine relaxin analogue consisting of the A (4-22) and B (1-23)NH₂ peptide chains.

21. A synthetic porcine relaxin analogue consisting of the A (4-22) and B[N-acetyl](4-23)NH₂.

22. A porcine relaxin analogue which has been modified by one or more of the following procedures:

(a) the addition of one or more amino-acids to one or

both of the natural A and B chains;

(b) the deletion of one or more amino acids from the natural B and/or A peptide chains;

(c) the chemical modification of one or more amino acids

(d) the replacement of at least one of the natural amino-acids in one or both of the A and B chains with a different amino acid

of the natural B and/or A peptide chains;

with the proviso that the thus modified porcine relaxin analogues possess porcine relaxin activity.

23. An analogue as claimed in claim 22 wherein

1

the chemical modification comprises the addition of a protective group to a free amino group.

DATED this 26th day of February, 1987
HOWARD FLOREY INSTITUTE OF EXPERIMENTAL
PHYSIOLOGY AND MEDICINE
by its Patent Attorneys
DAVIES & COLLISON

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[Handwritten signature]

A CHAIN

1 5 10

H-ARG-MET-THR-LEU-SER-GLU-LYS-CYS-CYS-GLN-VAL-GLY-CYS

15 20 22

ILE-ARG-LYS-ASP-ILE-ALA-ARG-LEU-CYS-OH

B CHAIN

1 5 10

PCA-SER-THR-ASN-ASP-PHE-ILE-LYS-ALA-CYS-GLY-ARG-GLU

15 20 25

LEU-VAL-ARG-LEU-TRP-VAL-GLU-ILE-CYS-GLY-SER-VAL-SER

28 29 31

TRP-GLY-ARG-THR-ALA-OH

FIGURE 1

0100 00 11 00

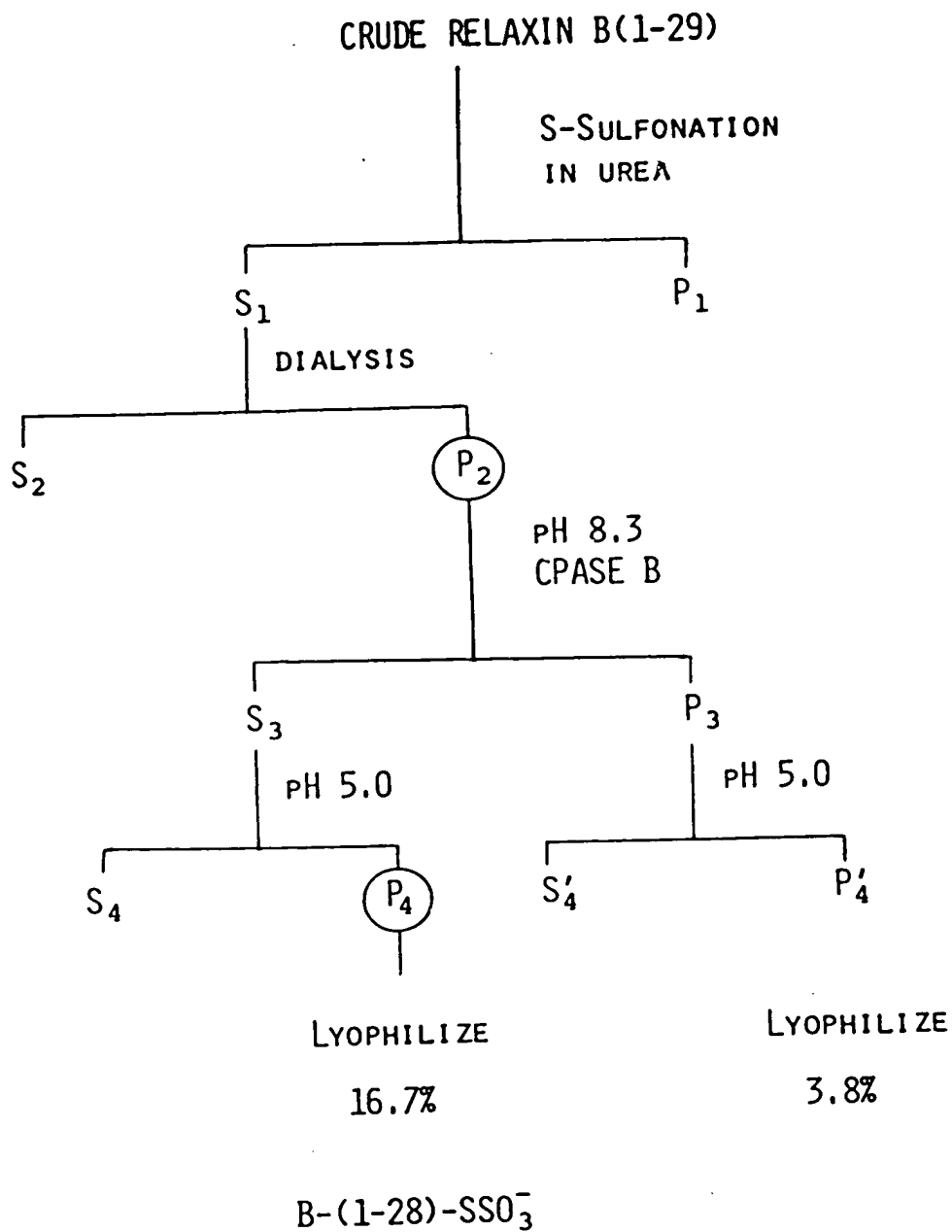


FIGURE 2



Wavelength nm

A 5x5 grid of dots forming a stylized letter 'A'. The dots are arranged as follows: Row 1: (1,1), (1,2), (1,4), (1,5); Row 2: (2,1), (2,2), (2,3), (2,4), (2,5); Row 3: (3,2), (3,3), (3,4); Row 4: (4,1), (4,2), (4,3), (4,4), (4,5); Row 5: (5,1), (5,2), (5,3), (5,4), (5,5).

A 4x4 grid of dots forming a stylized letter 'A'. The top row has dots at (1,1), (1,3), (1,4), (2,1), (2,3), (2,4), (3,1), (3,3), (3,4), (4,1), (4,3), (4,4). The middle row has dots at (1,2), (1,3), (1,4), (2,2), (2,3), (2,4), (3,2), (3,3), (3,4), (4,2), (4,3), (4,4).

$[\theta] \text{ MRW} \times 10^{-3} \text{ degree cm}^2 \text{ decimol}^{-1}$

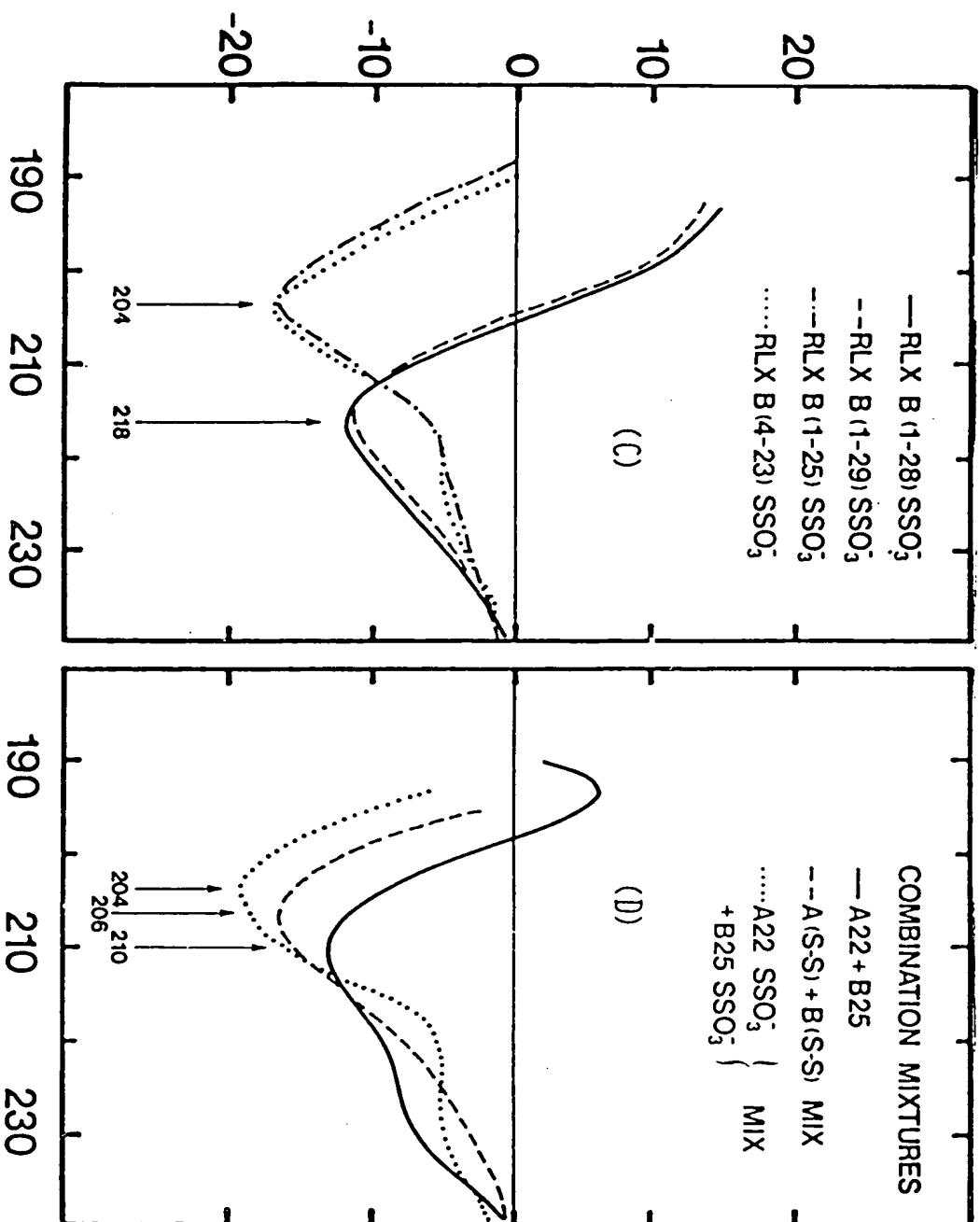
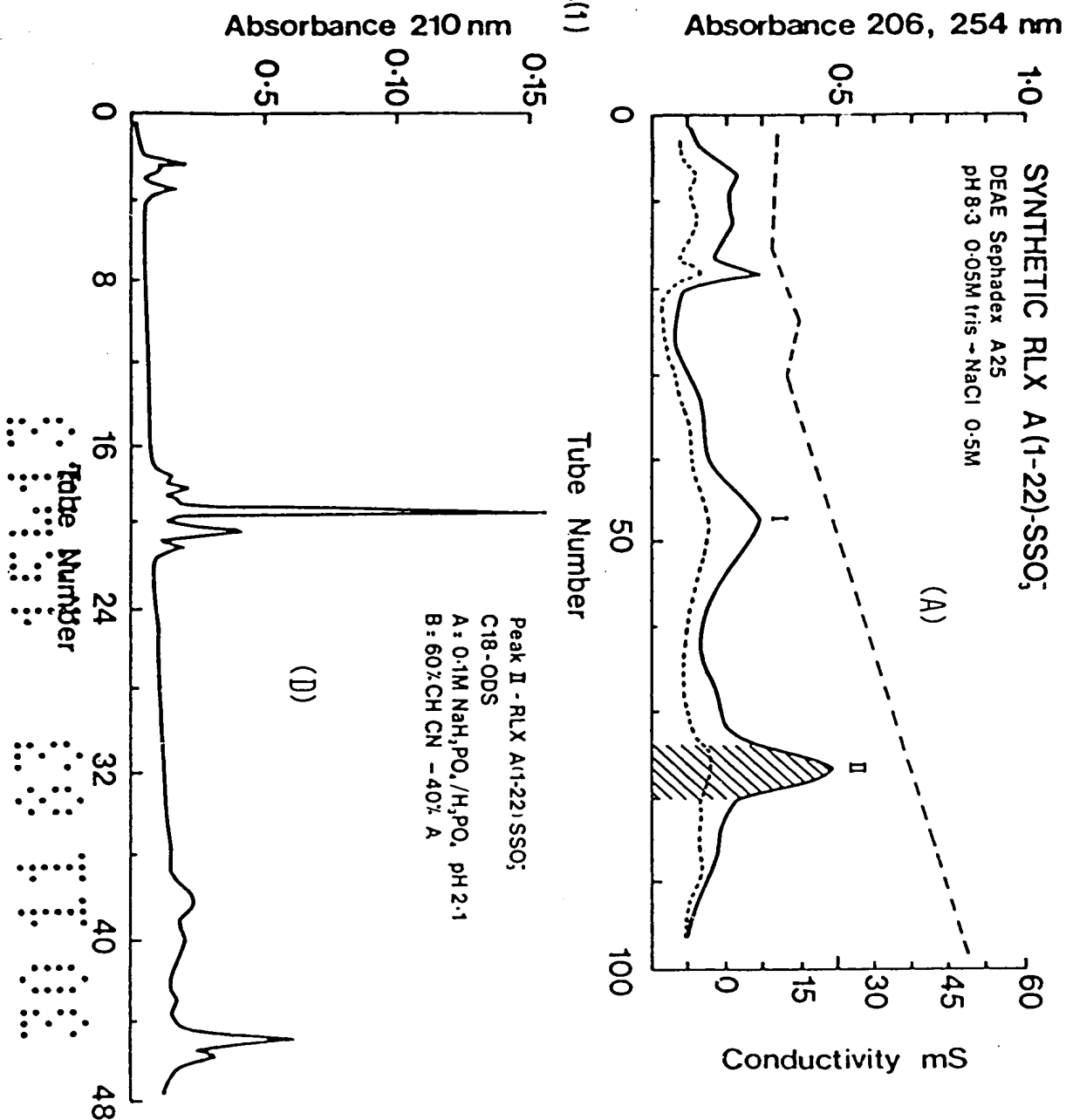


FIGURE 3(2)

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FIGURE 4(1)



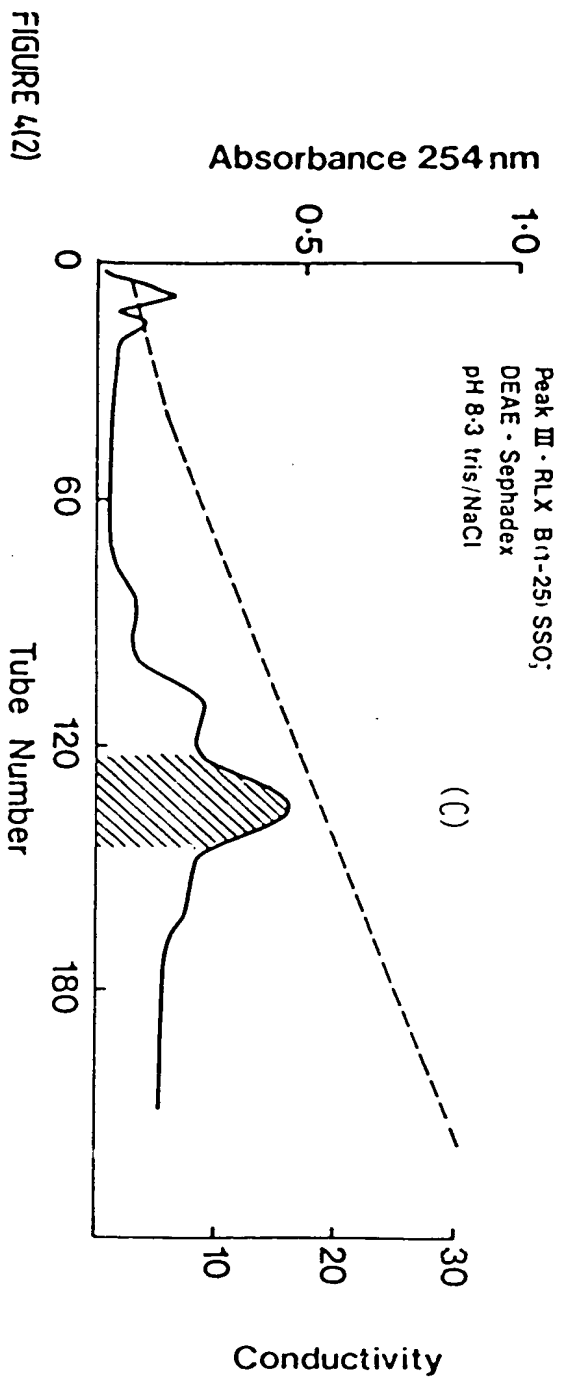
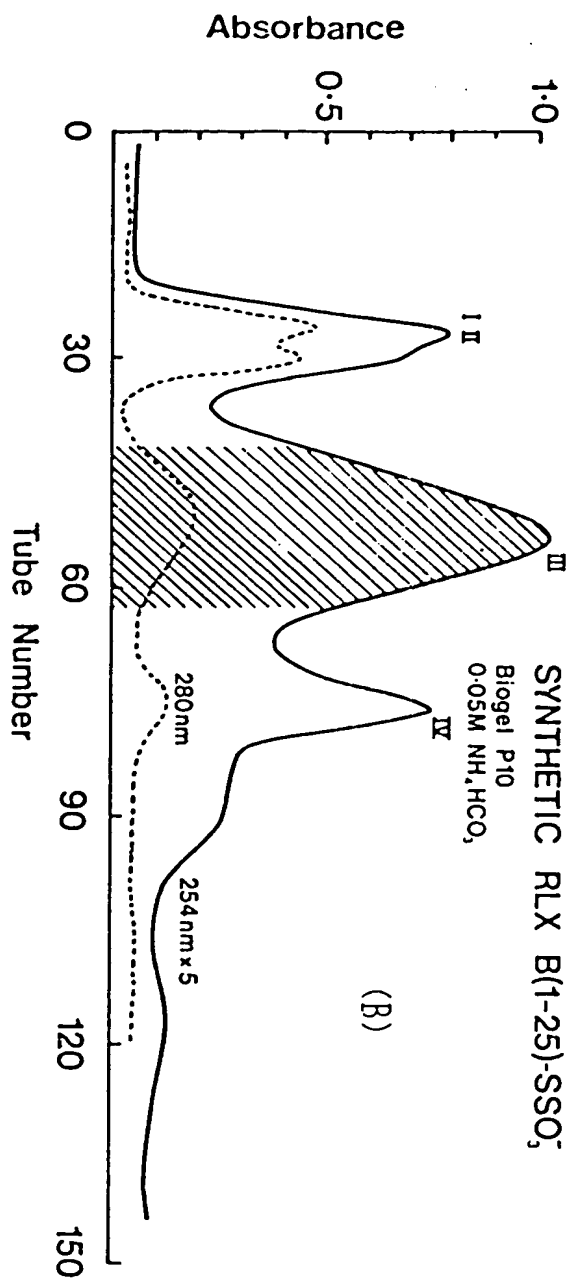


FIGURE 4(2)

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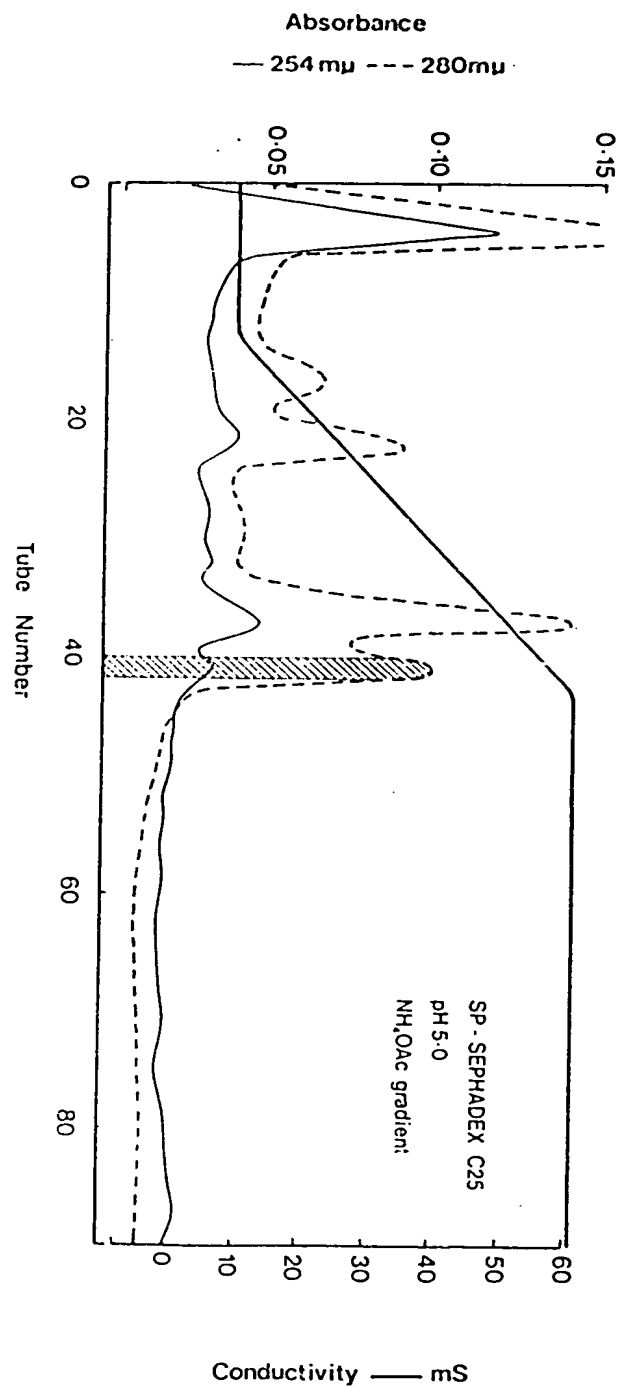


FIGURE 5

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